BOEING AEROSPACE COMPANY

P.O. Box 58747
Houston, Texas 77058

A Division of The Boeing Company

May 10, 1976
5-2510-HOU-2-1483

National Aeronautics and Space Administration
Goddard Space Flight Center
Greenbelt, Maryland 20771

Attention: Rhoda Schwartz
Contracting Officer/287.1

Subject: Contract NAS5-22545

Transmitted herewith is the Quarterly Report for the subject contract, covering the period January - March 1976.

If there are any questions, please contact the undersigned at (713) 488-0910, extension 209.

SPACE SYSTEMS DIVISION

Robert S. Anderson
Houston Contracts Manager

cc/w. enclosure:
Publication Branch/251
Patent Counsel/204
Technical Officer (7)/726

NASA CR

Enclosure to 5-2510-HOU-2-1483
dated May 10, 1976

Two Techniques for Eliminating Luminol Interference Material and Flow System Configurations for Luminol and Firefly Luciferase Systems

Prepared by:

RICHARD R. THOMAS
The Boeing Company
P. O. Box 58747
Houston, TX 77058

Approved by:

ROBERT H. NUSS
The Boeing Company
P. O. Box 58747
Houston, TX 77058

Quarterly Report for Period January - March 1976

Prepared for
Goddard Space Flight Center
Greenbelt, MD 20771

1. Report No. 2	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle: Two Techniques for Eliminating Luminol Interference Material and Flow System Configurations for Luminol and Firefly Luciferase Systems		5. Report Date 6. Performing Organization Code
7. Author(s): Richard R. Thomas		8. Performing Organization Report No.
9. Performing Organization Name and Address: The Boeing Company, P.O. Box 58747, Houston TX 77058		10. Work Unit No. 11. Contract or Grant No. NAS 5-22545 13. Type of Report and Period Covered: II - January-March 1976
12. Sponsoring Agency Name and Address: NASA/Goddard Space Flight Center, Grace Lee Picciolo, Code 726, Greenbelt, MD 20771		14. Sponsoring Agency Code
15. Supplementary Notes		

16. Abstract

Two methods for eliminating luminol interference materials are described. A method has been developed which eliminates interference from organic material. By pre-reacting a sample with dilute hydrogen peroxide organic materials can be inactivated. The reaction rate resolution method for eliminating inorganic forms of interference is also described. The combination of the two methods makes the luminol system more specific for bacteria.

Flow system designs for both the firefly luciferase and luminol bacteria detection systems are described. The firefly luciferase flow system incorporating nitric acid extraction and optimal dilutions has a functional sensitivity of 3×10^5 E. coli/ml. The luminol flow system incorporates the hydrogen peroxide pretreatment and the reaction rate resolution techniques for eliminating interference. The functional sensitivity of the luminol flow system is 1×10^4 E. coli/ml.

17. Key Words (Selected by Author(s))	18. Distribution Statement
Luminol, Interference, Luminol-Luciferase Flow System	

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price*
UNCLASSIFIED	UNCLASSIFIED		

* For sale by the National Technical Information Service, Springfield, Virginia 22151.

TABLES

ILLUSTRATIONS

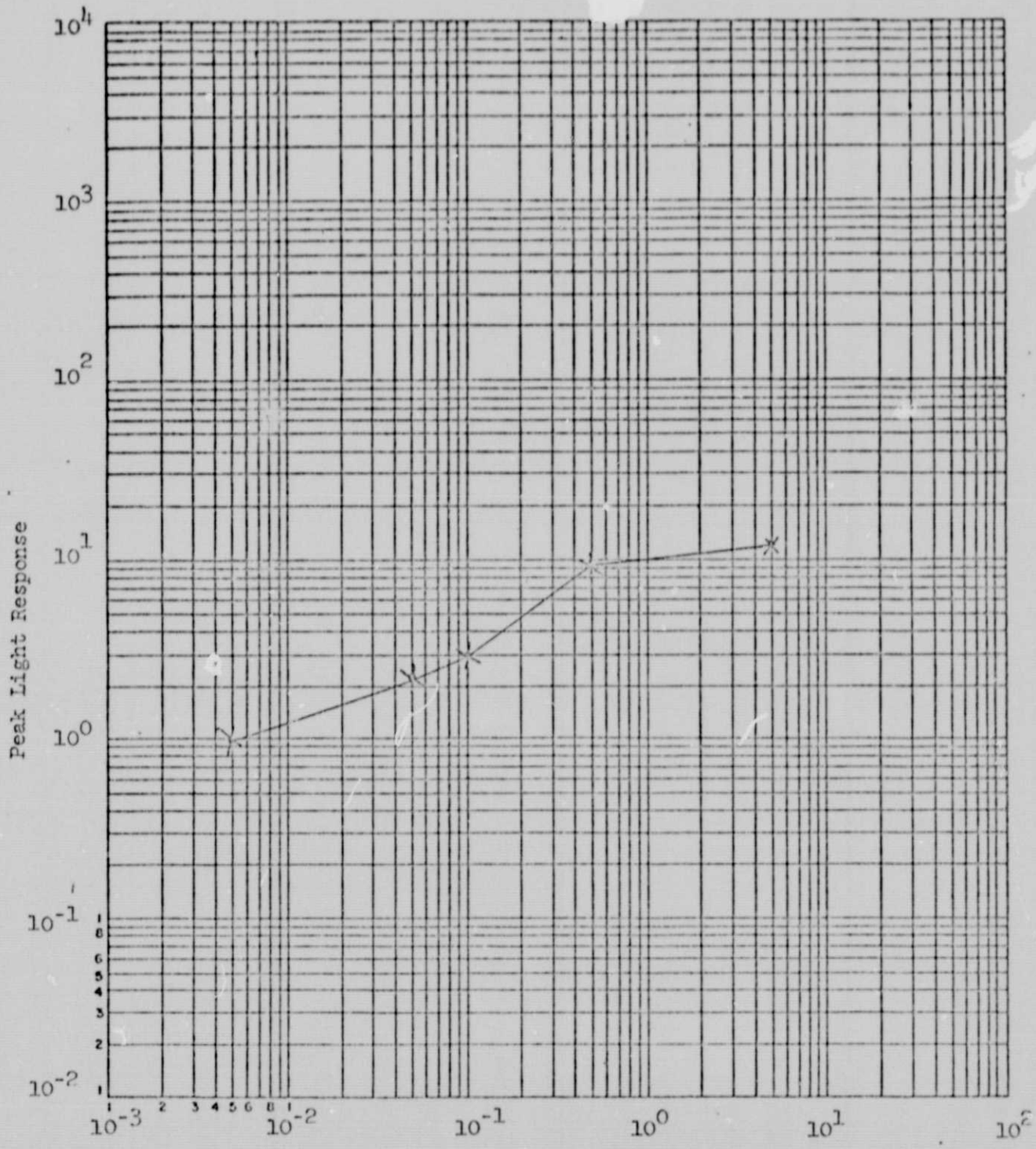
I. Hydrogen Peroxide

It was reported in Report No. 1[1] that sodium perborate produced a higher peak light response from the luminol reaction than did hydrogen peroxide. It has since been found that on a mole per mole basis, of all the hydrogen peroxide producing compounds, sodium perborate, sodium peroxide as well as hydrogen peroxide all produce the same light response for luminol oxidizing agents.

Figure 1 shows that the peak light response from luminol for a porphyrin sample is a function of hydrogen peroxide concentration. Although the peak light response varies with hydrogen peroxide concentration, the total light from a particular sample remains constant. Because of this, the shape of the curves for various peroxide concentrations vary from a sharp, high peak at high peroxide concentrations to lower but broader curves at lower concentrations. This combined with the characteristic reaction rate curves for the various luminol oxidizing agents provide the basis for the reaction rate resolution method for eliminating interference.[2]

Of the three hydrogen peroxide producing compounds used in this laboratory, sodium perborate and sodium peroxide have the advantage of being relatively stable solids. Sodium perborate has the disadvantage of having a limited solubility in water, 0.1625M = 0.55%. Sodium peroxide is very soluble in water; however, in addition to producing hydrogen peroxide when dissolved, also produces sodium hydroxide. This limits its use as a pretreatment since the sodium hydroxide could damage the bacteria cells. Although it has a limited shelf-life, hydrogen peroxide is still the most convenient reagent to use for the luminol mixture. It is recommended that the luminol-peroxide reagent mixture not be used when over four hours old.[3]

Figure 1. Peak luminol light respons[illegible] X 10^{-9} M catalase with varying hydrogen peroxide concentr[illegible].



II. Hydrogen Peroxide Pretreatment for Eliminating Interference

A technique has been developed for eliminating organic luminol interferences such as catalase, hemoglobin, and other soluble extra-cellular porphyrins. This method involves a pre-incubation of the sample with a dilute concentration of hydrogen peroxide to pre-react and thus inactivate the organic interferences. The bacterial porphyrins in the intact cells are protected and only react with the luminol reagent after they have been ruptured by the sodium hydroxide in the luminol mixture.

Table I shows the effect of 0.5% hydrogen peroxide pretreatment on several compounds which could interfere with the luminol-bacteria detection system. The 10 minute pretreatment eliminated over 90% of the interfering signal from the porphyrin materials. Figure 2 shows the effect of hydrogen peroxide concentration and time on a catalase sample. The greatest reduction of signal takes place within the first ten minutes of the reaction. Concentrations greater than 0.1% are necessary for effective reduction of interference signal.

TABLE I. Effect of 0.5% Hydrogen Peroxide Pretreatment (10 minutes) on Luminol Oxidizing Agents

	Reduction of Peak Signal
Hemoglobin	95%
Catalase	94%
Extracted Bacterial Porphyrins	97%
Potassium Ferricyanide	50%
Cobalt (ous) Chloride	20%
Ferrous Sulfate	0

Figure 3 shows the effect of hydrogen peroxide concentration and time on a sample of stationary phase E. coli. At hydrogen peroxide concentrations less than 1.5%, no significant reduction of response from E. coli was observed. Table II shows that the age of the cells has a definite effect on the susceptibility of the cells to hydrogen peroxide pretreatment. Actively dividing cells such as found in log phase growth are more susceptible to reduction by hydrogen peroxide than cells in the stationary growth phase.

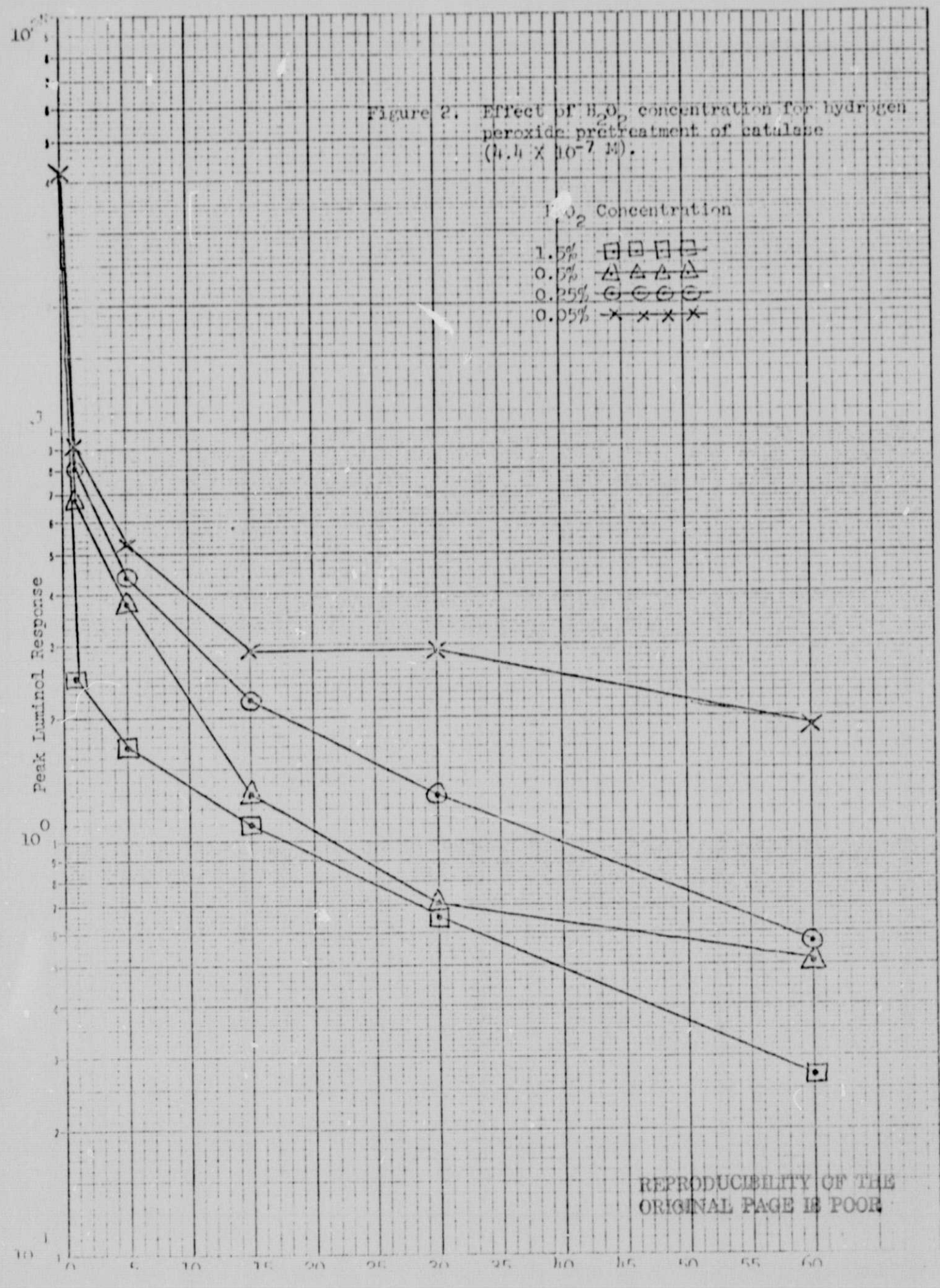


Figure 2. Effect of H_2O_2 concentration for hydrogen peroxide pretreatment of catalase (4.4×10^{-7} M).

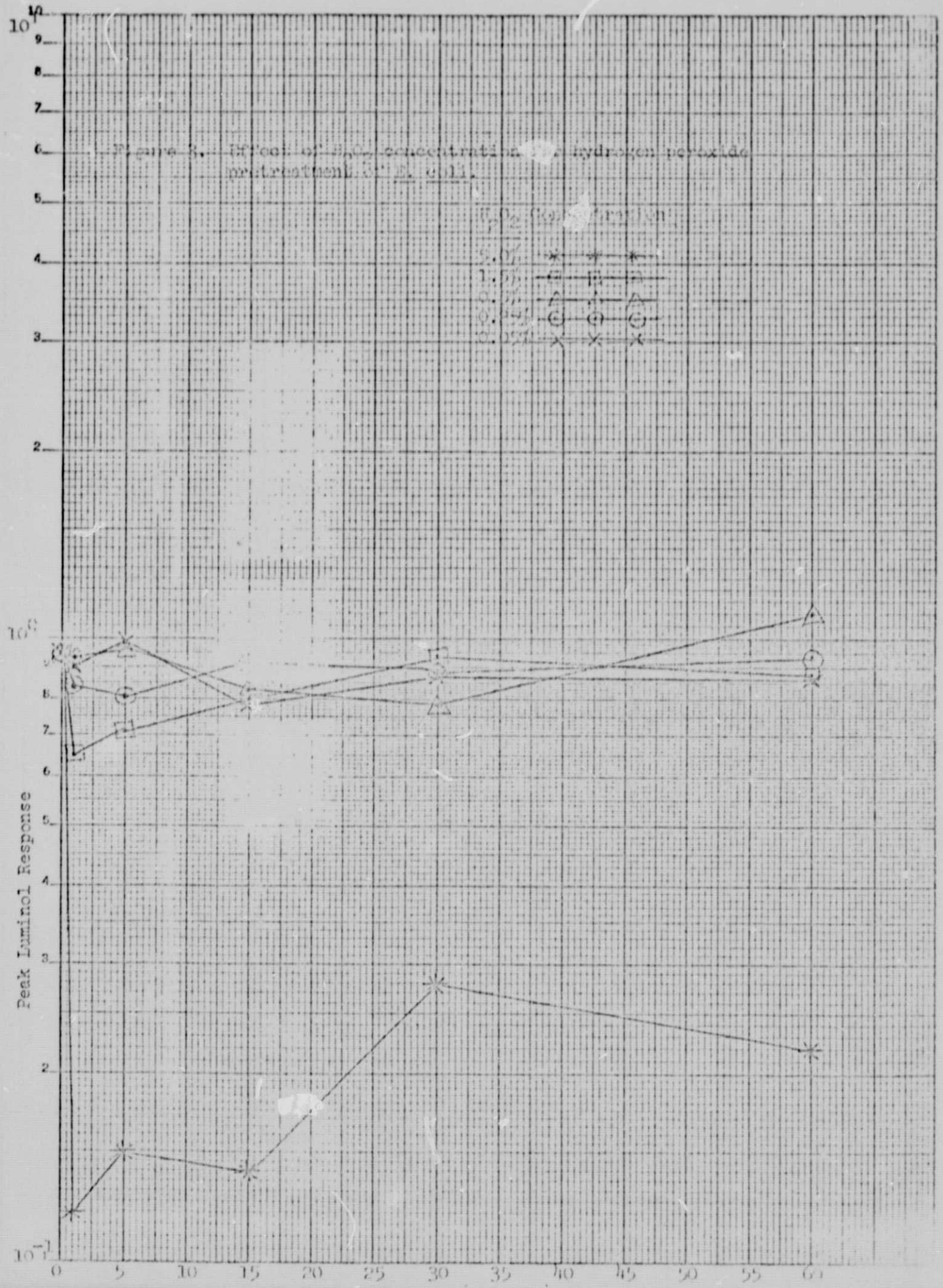


Figure 3. Effect of H_2O_2 concentration on hydrogen peroxide pretreatment of *E. coli*.

TABLE II. Effect of 0.5% Hydrogen Peroxide Pretreatment (10 minutes) on Bacteria.

	Reduction of Peak Signal	
	Growth Phase	
	Stationary	Log
Escherichia coli	8%	24%
Bacillis subtilis	16%	36%
Pseudomonas aeruginosa	43%	40%

Hydrogen peroxide is a more effective oxidant at higher pHs. By raising the pH of the 0.5% hydrogen peroxide pretreatment, the pretreatment is slightly more effective in eliminating soluble porphyrin interference while at the same time increasing the signal from the bacteria. Table III shows the effect of various buffered pH solutions of hydrogen peroxide and their effect on catalase and E. coli. As can be seen from the table 0.5% hydrogen peroxide buffered between pH 5.5 and 7.3 is most effective in eliminating catalase while decreasing the signal loss from E. coli. While a hydrogen peroxide pretreatment buffered around pH 7 is best for eliminating porphyrin interference and preserving the response from bacteria, the difference is not, however, great enough to warrant buffering the hydrogen peroxide pretreatment on a permanent basis.

TABLE III. Effect of Buffered 0.5% Hydrogen Peroxide Pretreatment on Catalase and E. coli.

		Reduction of Signal After 10 Minutes	
		10^{-8}M Catalase	E. coli
pH	Buffer		(log phase)
4.77	Citric acid	79%	40%
5.48	Citric acid	83%	27%
7.27	Phosphoric acid	83%	31%
9.19	Boric acid	87%	41%

In summary, the 0.5% hydrogen peroxide pretreatment for a period of ten minutes is the optimal condition for eliminating organic interference. Over 90% of soluble extra-cellular porphyrins can be eliminated using this technique.

III. Reaction Rate Resolution

As can be seen from Table I, the hydrogen peroxide pretreatment does not adequately eliminate inorganic forms of interference for the luminol reaction.

While there was a 20% reduction of luminol signal from pretreated cobalt(ous) chloride, no reduction of signal was noticed with ferrous sulfate. The ferrous sulfate may have already been oxidized to ferric sulfate when put into solution before assaying explaining the lack of a reduction in signal. To eliminate inorganic interferences of the luminol-bacteria system the reaction rate resolution method described in Report No. 1 must be used.[4] Figure 4 is an illustration of the type of reaction rate curves observed with several luminol oxidizing agents. When used in conjunction with the hydrogen peroxide pretreatment, the reaction rate resolution method can make the luminol system more specific for bacteria detection.

Table IV is data produced with the luminol flow system while at the Boeing Laboratory in Houston, Texas. The effect of each interference elimination method, both individually and together, on several water samples is shown. Overall 98% of the interference was eliminated from contaminated water samples.

TABLE IV. Luminol Response Following Interference Elimination Techniques.

	Relative Light Units			
	Deionized Water	Water Purified by Reverse Osmosis	Tap Water	*E. coli* Seeded Deionized Water
1) No pretreatment	0.49	17	41	27
2) H_2O_2 pretreatment	0.40	2.1	3.5	30
3) Reaction rate resolution	0.23	0.83	2.4	37
4) Combination of 2) and 3)	0.21	0.30	0.67	49
Overall Reduction of interference signal	57%	98%	98%	(+181%)

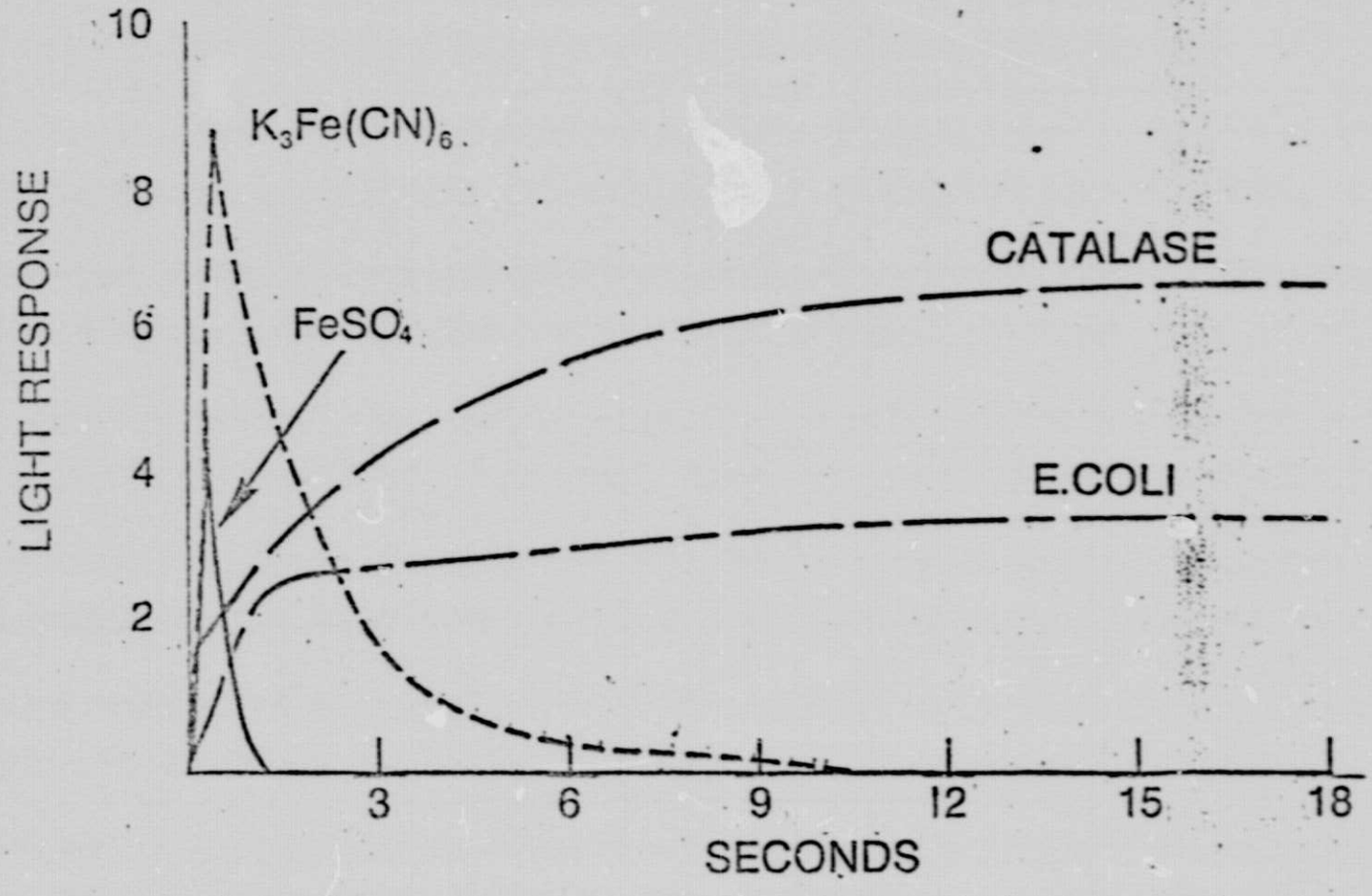


FIG. 4. REACTION RATE CURVES FOR VARIOUS LUMINOL OXIDIZING AGENTS (ARBITRARY CONCENTRATIONS).

IV. Flow System Configurations

A schematic diagram of the luminol flow system is shown in Figure 5. This system incorporates the two methods for eliminating interference problems. The hydrogen peroxide pretreatment allows the sample to pre-react the organic interferences and 0.4% hydrogen peroxide for a period of 2 minutes. The sample then reacts with the luminol reagent (2.5×10^{-4}M luminol, 0.1% H_2O_2 and 0.75 N $NaOH$) for a period of 10 seconds before reaching the photomultiplier tube. This step, reaction rate resolution, eliminates the interference from inorganic materials. The peak height is then measured to determine the bacteria concentration. Figure 6 shows the luminol flow system response to E. coli. The functional sensitivity of the system is 1×10^4 E. coli/ml.

The firefly luciferase flow system for detecting bacterial ATP has been constructed according to Figure 7. The flow system incorporates the optimal extraction and assay methods while requiring minimum dilution of the sample. The extraction step requires a 60 second residence time of sample with 0.1 N nitric acid and then subsequent dilution with water. The actual assay requires 0.2 ml of luciferase. Figure 8 is an E. coli concentration curve obtained with the flow system using DuPont luciferase (1 vial brought up to 3 ml with 0.25M TRIS, 10^{-2}M $MgSO_4$ and 10^{-3}M Cleland's reagent). The graph compares the response when measured as peak height and when the signal is integrated for a period of 60 seconds. While the sensitivity is the same in both cases, about 3×10^5 cells/ml, better linearity is achieved with the integrated signal.

The sensitivity of the flow system is about one-half the sensitivity of the injection system. This difference has been attributed to the configuration of the flow cell. The walls of the flow cell are much thicker than the walls of the cell used for the injection system and apparently absorb some of the light produced in the reaction.

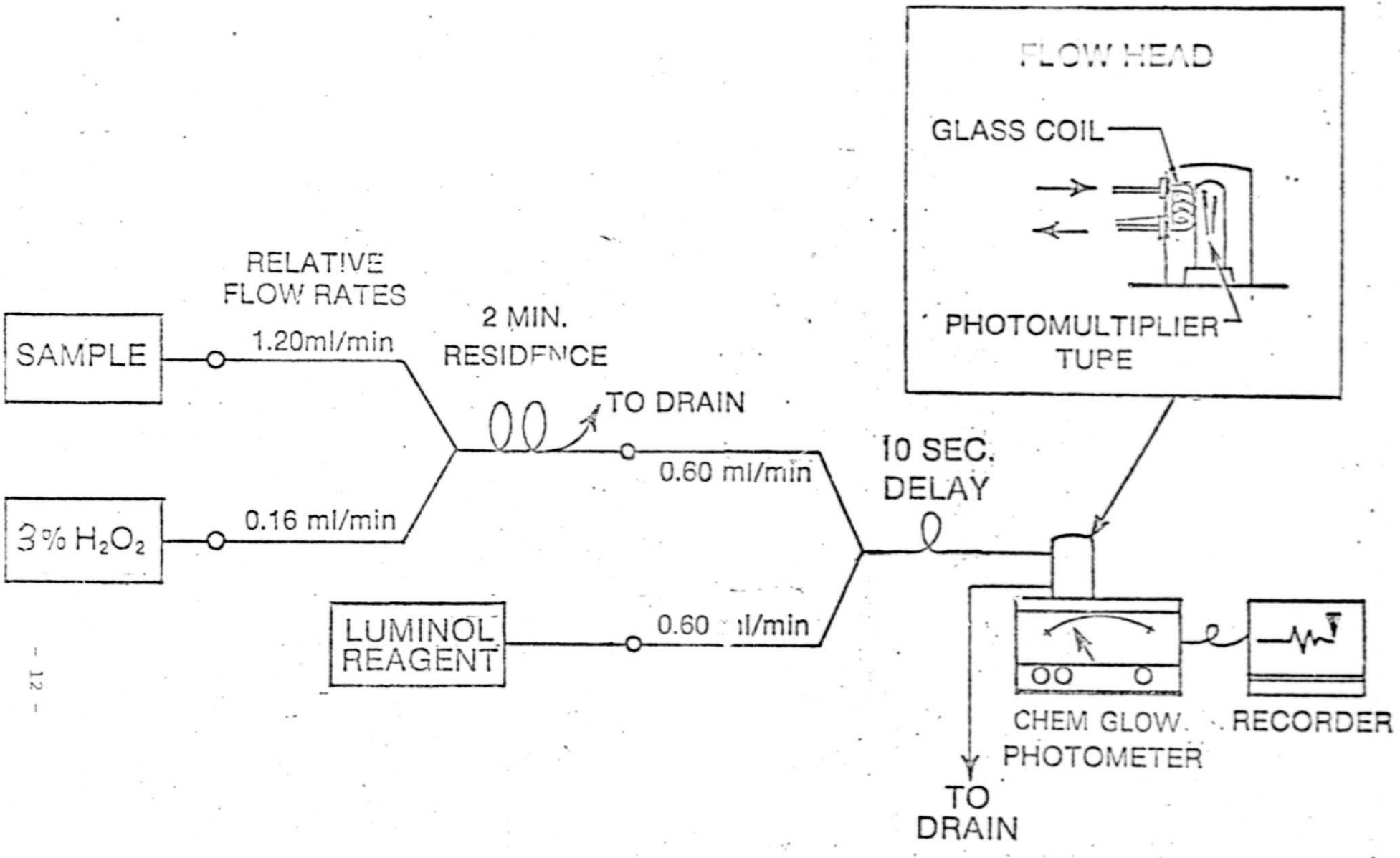
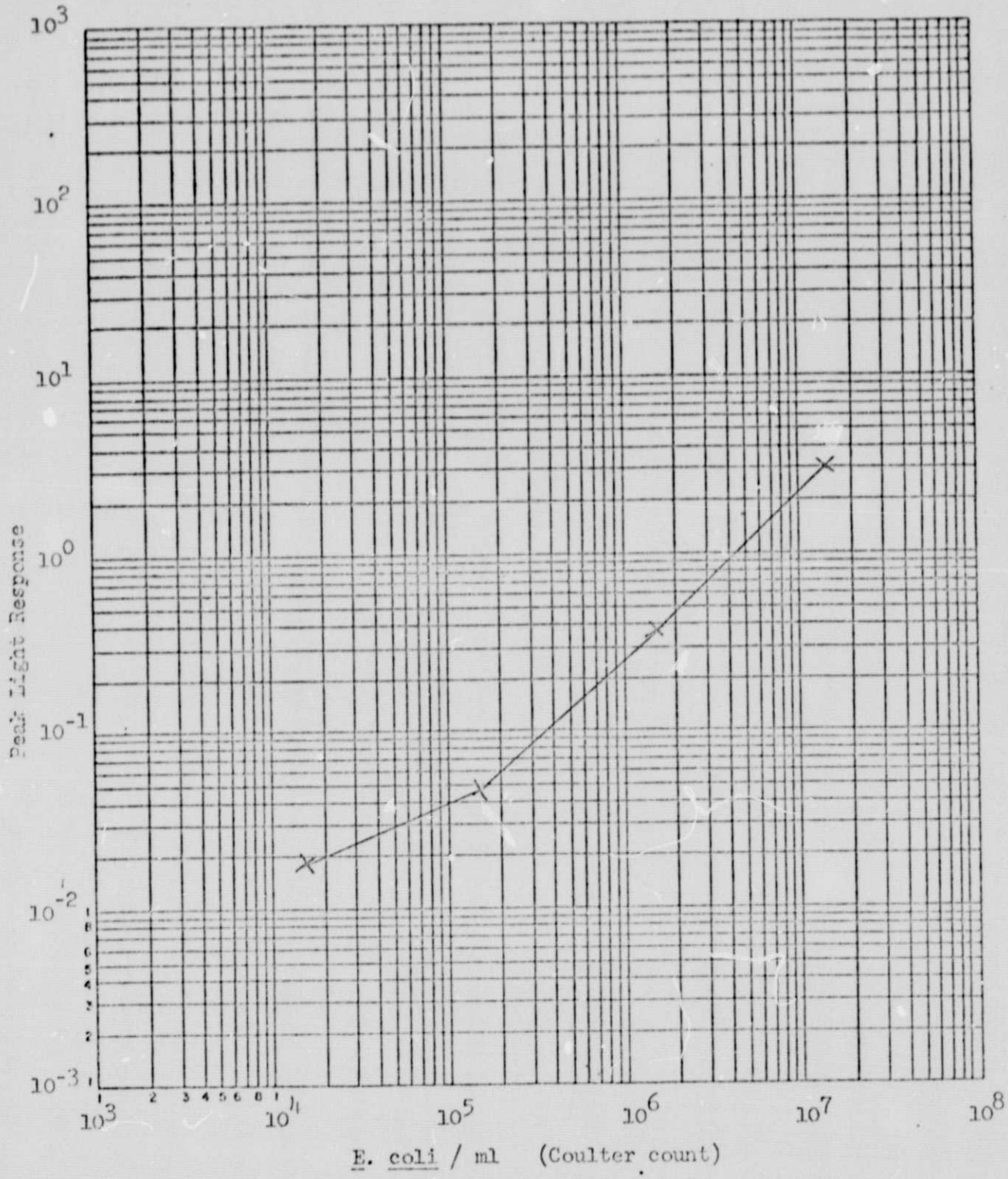


FIG. 5. AUTOMATED LUMINOL FLOW SYSTEM INCORPORATING HYDROGEN PEROXIDE PRETREATMENT AND REACTION RATE RESOLUTION FOR ELIMINATING INTERFERENCE

Figure 6. Peak light response from various concentrations of washed E. coli using the luminol flow system.



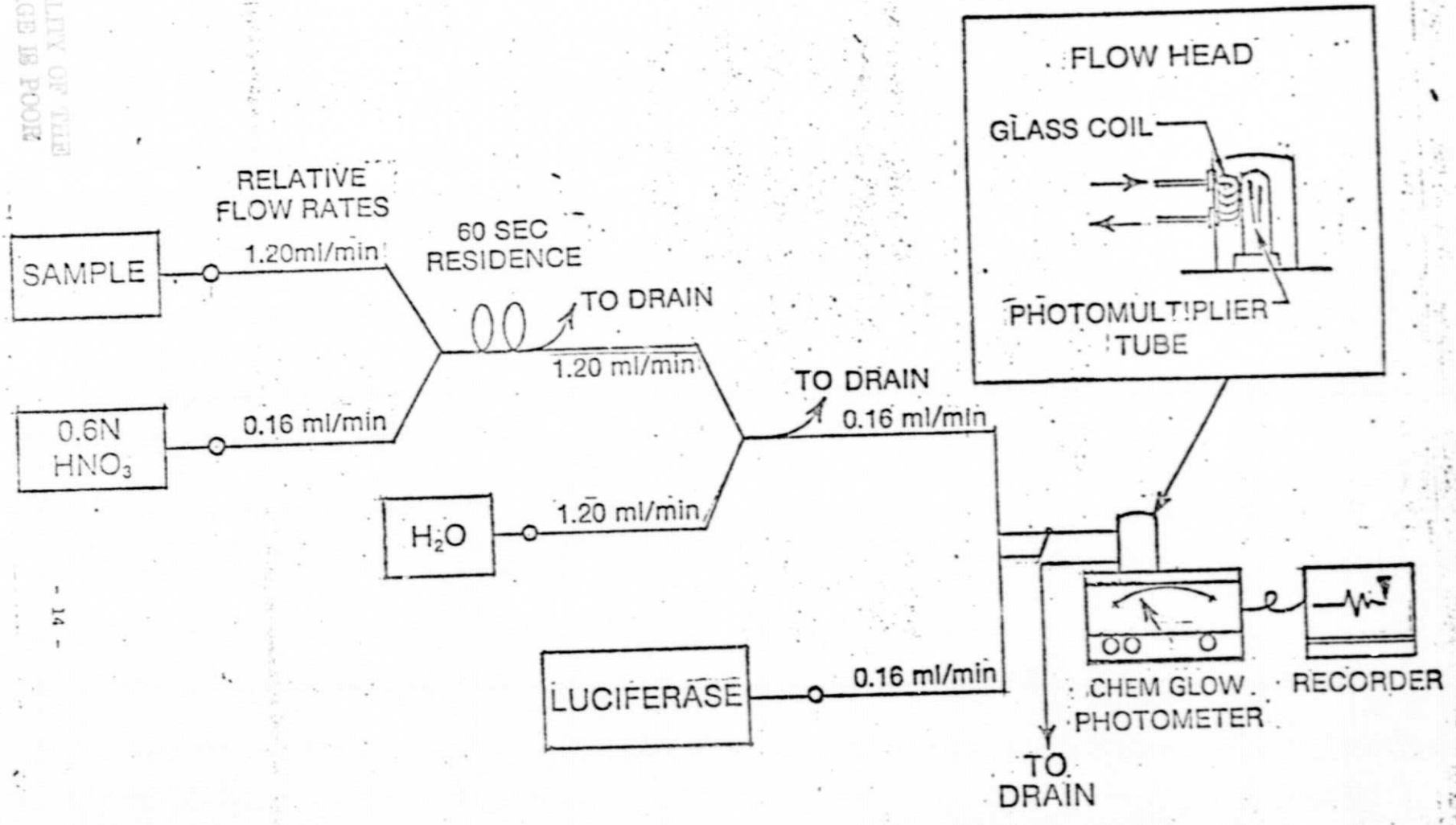
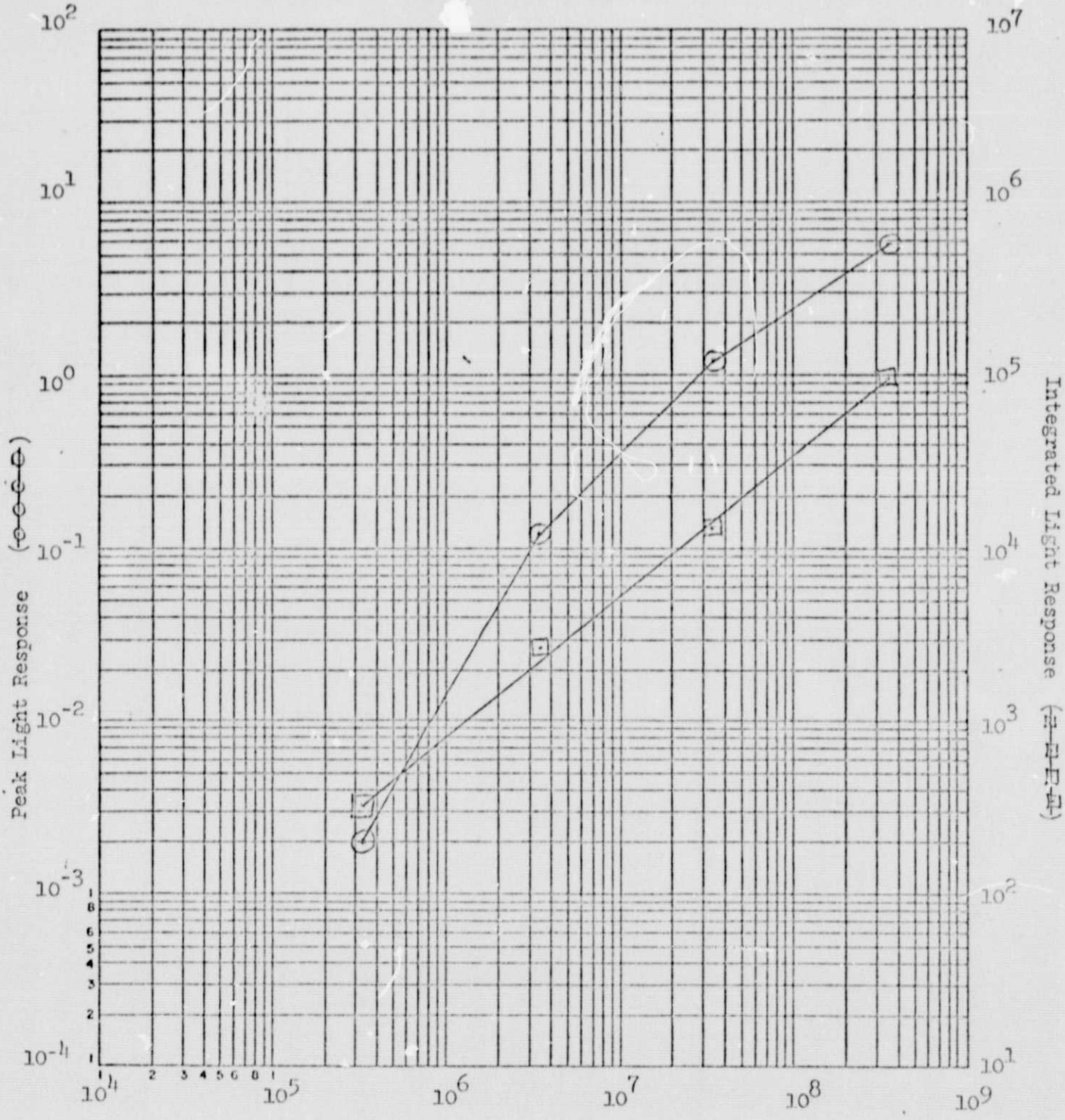


FIG. 7. AUTOMATED FIREFLY LUCIFERASE FLOW SYSTEM FOR DETECTING BACTERIAL ATP INCLUDING NITRIC ACID EXTRACTION AND SUBSEQUENT DILUTION

Figure 8. Light response from various concentrations of washed E. coli using the firefly luciferase flow system.



REFERENCES:

1. R. R. Thomas, "Development of a Chemiluminescent and Bioluminescent System for the Detection of Bacteria in Wastewater Effluent", Quarterly Report No. 1 to NASA; Contract No. NAS 5-22545, 1975, p. 9.

2. Ibid., p. 16.

3. Personal communication with Dr. Norma D. Searle, 13 February 76.

4. Quarterly Report No. 1 to NASA, p. 16.